EXHIBIT F

able variations in detection sensitivity and quantification of fetal DNA concentrations were noted among laboratories very familiar with real-time PCR technology *(21)*.

Further studies will be needed to clarify this issue in addition to the continued exploration of other strategies for the investigation of complex fetal genetic traits by analysis of maternal plasma. These strategies may include the enrichment of fetal sequences by size-fractionation of plasma DNA *(22, 23)* or the use of mass spectroscopy *(24)*, which has recently been shown to permit reliable detection of fetal point mutations.

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Lack of Dramatic Enrichment of Fetal DNA in Maternal Plasma by Formaldehyde Treatment, *Grace T.Y. Chung,[1†] Rossa W.K. Chiu,[1†] K.C. Allen Chan,[1] Tze K. Lau,[2] Tse N. Leung,[2] and Y.M. Dennis Lo[1*]* (Departments of [1] Chemical Pathology and [2] Obstetrics and Gynaecology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong Special Administrative Region, China; † these authors contributed equally; * address correspondence to this author at: Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, 30-32 Ngan Shing St., Shatin, New Territories, Hong Kong Special Administrative Region, China; fax 852-2194-6171, e-mail loym@cuhk.edu.hk)

The discovery of fetal DNA in maternal plasma has opened up new possibilities for noninvasive prenatal diagnosis *(1–6)*. A recent report *(7)* indicated that the proportion of fetal DNA in maternal plasma can be dramatically enriched through the adoption of a blood-processing protocol involving the addition of formaldehyde to maternal blood samples. Dhallan et al. *(7)* suggested that these observations might be the result of several factors, including a reduction in background maternal DNA by minimization of maternal cell lysis through formaldehyde-mediated cell membrane stabilization and the use of a gentle centrifugation protocol, as well as the preservation of fetal DNA through nuclease inhibition by formaldehyde.

In view of the profound implications of the study *(7)*, we aimed to validate and investigate the underlying mechanisms of the reported phenomenon. To assess the effects of several contributory factors, our study was conducted in three successive stages. In the first part of the study, we aimed to verify the effects of the previously published protocol *(7)* on total DNA concentrations in plasma from nonpregnant individuals. In the second part of the study, we evaluated the effects of formaldehyde addition on total and fetal DNA concentrations in maternal plasma in relation to the time of blood processing (0, 6, and 24 h after blood collection). In the last part of the study, we investigated whether the reported enrichment in circulating fetal DNA concentrations *(7)* might be a consequence of the imprecision of the analytical method chosen by the authors.

All participants were recruited with informed consent

from the Prince of Wales Hospital, Hong Kong, with institutional ethics approval. In the first part of the study, blood (24 mL) was collected into 3-mL EDTA tubes from each of eight nonpregnant volunteers and divided into four equal portions immediately after venesection. One portion was processed by a previously described two-step centrifugation protocol *(8)*, and the remaining three portions were centrifuged by the gentle centrifugation protocol described in the previous formaldehyde study *(7)*. One of the latter three portions was not treated further, whereas formaldehyde was added to the other two portions before or after centrifugation. For every 3 mL of blood, we added 75 μL of 10% neutral-buffered solution containing formaldehyde (40 g/L). This evaluation protocol was designed to assess whether the gentle centrifugation protocol and formaldehyde addition each contributed independently to the minimization of cell lysis and, thus, the decrease in total plasma DNA concentration. Plasma supernatants were carefully removed, and DNA was isolated by use of a QIAamp DNA Blood Midi Kit (Qiagen) and subjected to real-time quantitative PCR (RQ-PCR) for *β-globin (9)* on a 7900 Sequence Detector (Applied Biosystems).

Statistical analyses were carried out using Sigma Stat 3.0 (SPSS).

For plasma samples obtained immediately after venesection, *β-globin* DNA concentrations did not differ significantly among portions processed by the centrifugation protocols with or without formaldehyde addition before or after centrifugation ($P = 0.615$, Friedman; Table 1). As previous studies reported that circulating DNA concentrations would increase substantially after undue delays in blood processing *(10, 11)*, the second part of the study was designed to investigate the interrelationships between formaldehyde addition, plasma DNA concentrations, and delayed blood processing in pregnant women.

Pregnant women carrying male fetuses as identified by fetal ultrasound scanning were recruited for part 2 of the study. Blood (12 mL) was collected into 3-mL EDTA tubes from each of 13 and 17 pregnant women in the second (gestational age, 17–20 weeks) and third (gestational age, 36–41 weeks) trimesters of pregnancy, respectively. Portions of the whole blood specimens were centrifuged either immediately or after 6 or 24 h of storage at 4 °C. One half of each portion was treated with formaldehyde. Plasma *SRY* and *β-globin* DNA was quantified by RQ-PCR *(9)*. We compared the differences among the *SRY*, *β-globin*, and fractional fetal DNA concentrations in the maternal plasma aliquots processed at the three different time-points with or without formaldehyde addition sta-

Table 1. Summary of plasma DNA concentrations for parts 1 and 2 of the study.

	Median (IQR)[a]		
	***β-globin*, GE/mL of plasma**	***SRY*, GE/mL of plasma**	**Fetal DNA, %**
Part 1. Total plasma DNA in nonpregnant volunteers			
Two-step centrifugation protocol *(8)*	69.4 (62.7–124.2)	NA[b]	NA
Gentle centrifugation advocated by Dhallan et al. *(7)*	102.7 (66.9–142.4)	NA	NA
Formaldehyde added to plasma before gentle centrifugation	86.6 (70.8–136.3)	NA	NA
Formaldehyde added to plasma after gentle centrifugation	84.2 (62.5–115.8)	NA	NA
Part 2A. Maternal plasma total and fetal DNA in second-trimester pregnancies			
At 0 h			
No formaldehyde addition	795.4 (501.3–866.7)	25.0 (19.5–37.6)	4.0 (3.1–4.9)
Formaldehyde added	637.7 (464.6–1024.4)	29.5 (21.1–39.4)	5.0 (2.9–6.1)
At 6 h			
No formaldehyde addition	798.4 (726.5–1320.6)	23.7 (18.1–41.1)	2.7 (2.1–3.8)
Formaldehyde added	690 (588.1–918.9)	34.8 (28.7–47.4)	4.8 (3.4–6.4)
At 24 h			
No formaldehyde addition	1092.6 (804.7–1245.5)	29.4 (20.7–47.8)	3.3 (2.3–4.3)
Formaldehyde added	743.1 (607.8–898.2)	34.9 (21.5–45.2)	5.0 (3.5–5.6)
Part 2B. Maternal plasma total and fetal DNA in third-trimester pregnancies			
At 0 h			
No formaldehyde addition	1295.6 (787.3–1858.6)	140.8 (116.9–161.0)	9.9 (7.5–12.6)
Formaldehyde added	1303.1 (741.5–1695.4)	135.9 (86.6–248.1)	11.8 (8.5–15.3)
At 6 h			
No formaldehyde addition	1532.5 (754.4–2029.2)	143.9 (103.8–191.8)	9.1 (8.0–13.3)
Formaldehyde added	1139.6 (737.6–2292.0)	132.3 (96.9–232.9)	11.3 (9.1–14.7)
At 24 h			
No formaldehyde addition	1912.3 (1075.3–2402.0)	148.5 (86.0–189.1)	8.8 (5.7–12.6)
Formaldehyde added	1172.0 (607.8–1559.4)	113.4 (80.2–178.3)	12.1 (9.6–16.9)

[a] IQR, data range between the 25th and 75th percentiles of the dataset.
[b] NA, not applicable.

tistically were compared by Friedman repeated-measures ANOVA on ranks followed by all-pairwise post hoc analysis using the Dunn method to identify the specific groups that differed from the others.

The median *β-globin*, *SRY*, and fractional fetal DNA concentrations for the differently processed maternal plasma aliquots are summarized in Table 1. The maternal plasma *β-globin* concentrations for the different blood processing protocols were statistically different for samples from both the second ($P = 0.02$, Friedman) and third ($P = 0.002$, Friedman) trimesters of pregnancy. Post hoc analysis revealed that among all possible comparisons, the only significant difference was between the *β-globin* concentrations in the two third-trimester maternal plasma portions processed with 24-h delay with and without formaldehyde addition ($P < 0.05$, Dunn). The corresponding *β-globin* concentrations in the second-trimester plasma aliquots processed at 24 h demonstrated the greatest difference in ranks among all possible comparisons. However, this difference did not reach statistical significance in the post hoc analysis ($P > 0.05$, Dunn). We found no statistical difference between the maternal plasma *SRY* concentrations (Table 1) for any of the plasma aliquots in either the second ($P = 0.600$, Friedman) or third ($P = 0.697$, Friedman) trimesters of pregnancy. The plasma portions treated with formaldehyde demonstrated a small (1–3.3% increase in median values; Table 1) but nonsignificant increase in the fractional fetal DNA concentrations at each of the time-points in both the second ($P = 0.237$, Friedman) and third trimesters ($P = 0.339$, Friedman) of pregnancy (Table 1). These data contrasted markedly with the extent of fetal DNA enrichment reported previously *(7)* (59% of samples with fetal DNA percentages >25% with a range extending to >50%).

To investigate the cause of the discrepancy, we compared the analytical performance of RQ-PCR *(9)* with the serial dilution method used in the original formaldehyde study *(7)* for quantification of maternal plasma DNA. Blood (12 mL) was collected from two pregnant women carrying male fetuses (gestational age, 17 and 18 weeks). Formaldehyde was added, and plasma was obtained by gentle centrifugation *(7)*. A solution containing 4.5% male DNA [20 genome-equivalents (GE) of *SRY* among 450 GE of *β-globin*] was also prepared by mixing male and female genomic DNA. Extracted DNA from the three specimens was separated into 10 aliquots and assayed for *SRY* and *β-globin*. The fetal DNA percentage of each aliquot (30 aliquots in total) was determined by both the RQ-PCR method [*SRY* (GE/mL)/*β-globin* (GE/mL) × 100] *(9)* and the method based on determining the highest dilutions of input DNA for which the *SRY* and *β-globin* amplicons were detectable by gel electrophoresis *(7)*. Six serial 1:5 dilutions (1:5 to 1:15 625) were made for all aliquots.

Ten-replicate analyses of the artificial male DNA mixture yielded median [mean; interquartile range (IQR)] fetal DNA percentages of 6.3% (6.7%; 6.0–7.3%) by RQ-PCR and 8.0% (15.7%; 1.6–40%) by serial dilution (Fig. 1). The results based on the latter method were particularly

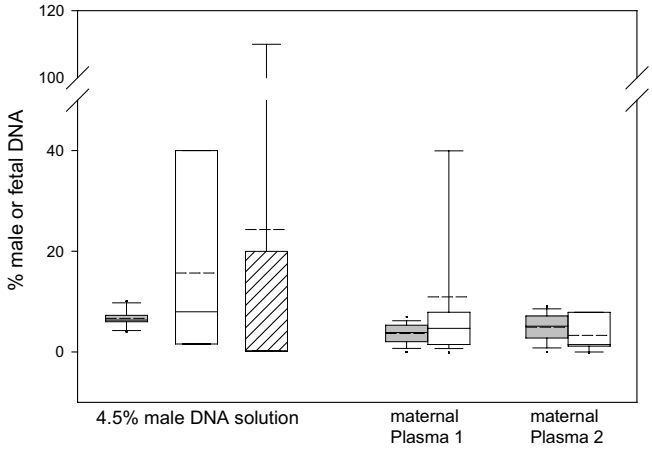

Fig. 1. Box plots of fractional male/fetal DNA concentrations determined by 10-replicate analysis by RQ-PCR (▨) and by the semiquantitative method with 5-fold (□) or 10-fold (▨) dilutions.

The *solid lines inside* the *boxes*, the *upper* and *lower limits* of the *boxes*, and the *whiskers* indicate the medians, 75th and 25th, and 90th and 10th percentiles, respectively. The *dashed lines* indicate the means. The upper whiskers are not plotted when the 90th and 75th or the 10th and 25th percentiles coincided. The median for the group represented by ▨ coincided with the lower limit of the box, 0.2%.

noteworthy in that they (*a*) deviated more from the expected 4.5% than that determined by RQ-PCR; (*b*) exhibited a wide discrepancy between the mean and median; and (*c*) demonstrated large fluctuations on repeated measurements as evident by the wide IQR (Fig. 1). In fact, these were signs of inaccuracy, skewed data distribution, and analytical imprecision, respectively.

A priori, RQ-PCR is likely to be more accurate and precise than the serial dilution method because male DNA percentages can be expressed as any value between 0% and 100% by RQ-PCR whereas, based on a fivefold dilution series, male DNA percentages can be scored only as one option among a discrete set of percentages, i.e., 0.0128%, 0.064%, 0.32%, 1.6%, 8%, or 40%. Because of the categorical nature of this scoring method and the uneven numerical distribution of the categories, the data would be skewed and inaccurately represented. For example, fetal DNA concentrations between 8% and 40% may only be scored as 8% or be promoted to the next category, namely, 40%. In fact, this phenomenon was also observed from the datasets presented in the previous formaldehyde study *(7)*. Using a twofold dilution series, the authors reported fetal DNA concentrations of >50% in several maternal plasma samples [see Table 2 in Dhallan et al. *(7)*]. By virtue of the twofold dilution, a fractional fetal DNA concentration >50% would essentially be scored as 100%.

In addition, the analytical result and the performance of the serial dilution method can be a function of the arbitrary choice of the dilution factor. Although previous work discussed above used 2- and 5-fold dilutions, the effect of large dilution factors can be illustrated by considering 10-fold dilutions. The artificial DNA mixture was additionally analyzed by 10-replicate measurements by a series of four 10-fold dilutions, where the male DNA

percentage can be expressed only as 0.02%, 0.2%, 2%, or 20%. As expected, the male DNA percentages deviated further from the expected (median, 0.2%; mean, 24.3%; IQR, 0.2–20%), with the skewness becoming more severe (Fig. 1). These data could be interpreted in completely opposite ways depending on whether the median (much lower than the true value of 4.5%) or the mean (much higher than the true value of 4.5%) was chosen for presentation.

The issue associated with analytical imprecision was equally applicable to the estimation of fetal DNA percentages. Repeated measurements of the two second-trimester maternal plasma samples by RQ-PCR yielded a median of 3.9%, mean of 3.7%, and IQR of 2.1–5.3% and a median of 5.2%, mean of 5.0%, and IQR of 2.8–7.2%, respectively. However, results for the fivefold serial dilution method were as follows: median, 4.8%; mean, 11.1%; IQR, 1.6–8%; and median, 1.6%; mean, 3.4%; IQR, 1.6–8%, respectively (Fig. 1).

We therefore conclude that, in our hands, gentle centrifugation did not confer any observable advantage and formaldehyde addition did not yield the previously reported dramatic increases in fractional concentrations of fetal DNA *(7)*. The latter could have resulted from the imprecise estimation of the fetal DNA percentages in maternal plasma when the previously reported serial dilution method was used.

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Quantification of Thiol-Containing Amino Acids Linked by Disulfides to LDL, *Angelo Zinellu, Salvatore Sotgia, Luca Deiana, and Ciriaco Carru** (Chair of Clinical Biochemistry, University of Sassari, Viale San Pietro 43/B, 07100 Sassari, Italy; * author for correspondence: fax 39-079228120, e-mail carru@uniss.it)

Several studies have indicated that plasma proteins interact with homocysteine (Hcy) to form stable disulfide-linked products. Hcy in plasma is mainly bound to albumin, but interactions with ceruloplasmin, fibrin, annexin II, and transthyretin have been also reported *(1–4)*. In 1991, Olszewski and McCully *(5)* described the presence of Hcy in lipoproteins in patients with hypercholesterolemia. Because the analysis was performed after acidic hydrolysis of apoprotein, the Hcy measured by these authors was the sum of (*a*) Hcy incorporated in the primary structure of apolipoprotein B-100 *(5)*, (*b*) Hcy thiolactone bound to lysine residues of protein by amide or peptide linkages and converted to Hcy by acidic conditions after release *(6)*, and (*c*) Hcy linked to apolipoprotein B-100 (apoB-100) by a disulfide bond.

Recent studies have demonstrated that there are at least nine free sulfhydryl groups (−SH) in the apoB-100 primary structure that could potentially bind plasma free aminothiols by disulfide linkage *(7)*. Because lysyl residues of apoB-100 could react in vivo with plasma Hcy thiolactone by an amide bond, the number of free apoprotein sulfhydryl groups could increase considerably, thus increasing the number of sites that may be bound with plasma aminothiols *(6)*. These LDL modifications are accompanied by an increase in density and in electrophoretic mobility of lipoprotein and are associated with functional alterations that make Hcy-LDL more susceptible to aggregation and to spontaneous precipitation. Moreover, higher uptake of Hcy-LDL by membrane receptor and by phagocytosis and a higher accumulation of intracellular cholesterol have been observed in cultured macrophages, suggesting that homocysteinylation could increase the atherogenicity of LDL *(8, 9)*. Thus, to study the association between Hcy and lipid metabolism, a highly sensitive method to measure Hcy and other thiols bound to apoB-100 is required.

Here we describe a simple capillary electrophoresis method with laser-induced fluorescence detection to measure physiologic thiols bound to apoprotein by a disulfide linkage. We assessed the performance of this analytical method by measuring apoB-100-bound thiols in 16 volunteers. Participants were not receiving dietary supplements of vitamin B_6, B_{12}, or folate or statin therapy as deter-